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Lack of ceruloplasmin expression alters aspects of copper transport to the fetus and newborn, as determined in mice

Yu-Ling Chu · Eric N. Sauble · Anthony Cabrera · Aleeza Roth · M. Leigh Ackland · Julian F. B. Mercer · Maria C. Linder

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Abstract Copper transport and accumulation were studied in virgin and lactating C57BL/6 mice, with and without expression of ceruloplasmin (Cp), to assess the importance of Cp to these processes. One hour after i.p. injection of tracer ^{64}Cu, liver and kidney accounted for 80% of the radioactivity, and mammary gland 1%, while in lactating Cp+/+ mice 2–4 days post partum, uptake by mammary gland was 9-fold higher and that of liver and other organs was decreased, with ^{64}Cu rapidly appearing in milk. Parallel studies in Cp−/− mice (siblings from same colony) gave virtually identical results. However, their milk contained less ^{64}Cu, and actual copper contents determined by furnace atomic absorption were less than half those for milk from normal dams. Liver copper concentrations of pups born to Cp−/− dams also were half those of pups from wild type dams. Copper in pup brains was unaffected; but iron concentrations were reduced. We conclude that absence of Cp, while not affecting entry of exchangeable copper from the blood into the mammary gland, does have a significant effect on the availability of this metal to the newborn through the milk and in the form of stores accumulating in gestation.

Keywords Copper · Ceruloplasmin · Copper-64 · Milk · Mammary gland · Iron

Y.-L. Chu · E. N. Sauble · A. Cabrera · A. Roth · M. C. Linder (✉)
Department of Chemistry and Biochemistry, California State University, Fullerton, CA 92834-6866, USA
e-mail: mlinder@fullerton.edu

M. Leigh Ackland · J. F. B. Mercer
Centre for Cellular and Molecular Biology, Deakin University, Burwood, VIC 3052, Australia

Introduction

It is well known that during mammalian gestation, the fetal liver accumulates a store of copper, as well as iron and zinc (Linder 1991; Linder et al. 1999; Linder 2010). Offspring are born with very high liver copper concentrations, mainly bound to metallothionein in the nucleus and cytoplasm of hepatocytes (Cherian et al. 1987; Linder 1992; Nartey et al. 1987). These copper "stores" are utilized for infant growth during the suckling period, bringing liver concentrations down to those of the adult by the end of lactation (e.g. 3 weeks after birth in the case of rats and mice) (Linder 1991; Linder 2010). Additional copper, also needed for growth, is supplied through the milk. Thus, as shown in rats, the total copper content of the newborns increases markedly (6-8-fold in the 1st week after birth) during the period when milk is the only or main food (Linder 1991; Linder 2010).

In the normal course of events, copper entering the blood first binds to albumin and transcuprein (a macroglobulin), which constitute the exchangeable plasma copper pool (Linder 1991; Weiss and Linder

1985). Based on radioactive tracer studies in rats (Linder 1991; Weiss and Linder 1985; Owen 1971) and stable isotope studies in humans (Turnlund et al. 1998), most of this copper then enters the liver, where it is either shuttled to liver organelles and specific enzymes, excreted into the bile, and/or incorporated into ceruloplasmin for secretion back into the blood. The latter occurs in the trans Golgi network with the help of the copper pump, ATP7B (La Fontaine and Mercer 2007; Linder 2002; Lutsenko et al. 2007). As determined by size exclusion separation of plasma components and metal analysis, ceruloplasmin accounts for about two-thirds of the plasma copper (totaling about 1.2 μg/ml) in human and rat plasma (Barrow and Tanner 1988; Linder 1991; Wirth and Linder 1985), and about half that in the case of mice (which also have a much lower total – of about 0.4 μg/ml) (Cabrera et al. 2008; Gray et al. 2009). Ceruloplasmin-copper is buried within its structure (Zaitsev et al. 1999), not dialyzable (Linder 1991), and thus not exchangeable with copper on albumin and transcuprein, i.e. it is not part of the "exchangeable plasma copper pool". Nevertheless, earlier studies by our group indicated that plasma ceruloplasmin is the main normal source of copper transferred from the dam to the fetus, via the placenta, in gestation (Lee et al. 1993). When pregnant rats were infused intravenously with radioactive copper either bound to ceruloplasmin or to albumin and transcuprein, radioactivity in ceruloplasmin entered the placenta and fetus much more rapidly than that from the exchangeable plasma pool. Moreover, inhibition of ceruloplasmin synthesis with cycloheximide markedly reduced appearance of the radiotracer in plasma ceruloplasmin and was accompanied by a marked reduction in amounts of tracer in placenta and the fetuses, further corroborating the importance of the mother's ceruloplasmin for the fetus. Plasma ceruloplasmin appears to deliver copper not just to the placenta but also to a variety of other organs (Campbell et al. 1981; Hilton et al. 1995; Lee et al. 1993; Linder 1991; Linder 2002; Linder et al. 1999; Tong and McArdle 1995). Moreover, the evidence available suggests this may occur by interaction of ceruloplasmin with specific receptors that have been detected on the membranes of several kinds of cells, including those of the heart, skeletal muscle and placenta (Barnes and Frieden 1984; Hilton et al. 1995; Kataoka and Tavassoli 1985; Orena et al. 1986; Stevens et al. 1984; Tong and McArdle 1995).

Ceruloplasmin is also present in other bodily secretions, including amniotic fluid (ingested by the fetus during gestation) and milk (Cerveza et al. 2000; Linder et al. 1999). Milk ceruloplasmin is specifically made by the mammary epithelial cells (Cerveza et al. 2000; Wooten et al. 1996). In the mammary gland, too, it is likely that ATP7B is necessary for copper incorporation into ceruloplasmin (in the trans Golgi network), and for importing copper into the milk, since mutations that impair the activity of this copper pump (such as those of the toxic milk [tx] mouse) reduce milk copper concentrations about 6-fold (Michalczyk et al. 2000; Rauch 1983). We and some others have determined by immunoassays, copper radiolabeling and ceruloplasmin enzyme activity assays that up to about 25% of the copper in the post-colostrum milk of rats and humans is associated with ceruloplasmin (Cerveza et al. 2000; Donley et al. 2002; Kiyosawa et al. 1995). Platonova et al. (2007) contend that all of human milk copper is with ceruloplasmin, and that it is absorbed as the whole protein by newborn rats. Either way, milk ceruloplasmin is likely to be important for the copper nutrition of the newborn, and this is further supported by our finding that its copper was absorbed much better by rat pups than ionic copper added to milk (Wooten et al. 1996).

As already noted, albumin and transcuprein comprise the main or only components of the plasma exchangeable copper pool (Donley et al. 2002; Linder 1991; Linder 2010). Albumin is the most abundant plasma protein, accounting for about 70% of the total protein in plasma but only about 15% of the copper in human and rat plasma, where it binds to a high affinity site on the N-terminus. Transcupreins are macroglobulins (alpha-2-macroglobulin in most species; alpha-1-inhibitor 3 in rodents) (Liu et al. 2007), with even higher affinities for copper, and account for another 15% of human plasma copper. Copper bound to albumin and transcuprein usually enters the liver and kidney before reemerging in the blood circulation on ceruloplasmin (Linder 1991; Weiss and Linder 1985). However, we found that in lactation (at least in rats) a large proportion was diverted from liver and kidney directly to the mammary gland and rapidly entered the milk (Donley et al. 2002). Already 1 h after injecting traces of ^{67}Cu(II) into lactating Sprague–Dawley rats 2–5 days post-partum, about 50% was in the mammary gland, and a substantial proportion had already entered the milk. We thus wondered whether the same

phenomenon occurs in the mouse whose copper metabolism differs in some aspects (Cabrera et al. 2008; Gray et al. 2009).

Since the evidence described points to the special importance of ceruloplasmin as a source of copper for the fetus (via the placenta) and newborn (via milk), we were also interested in determining what would be the consequences if ceruloplasmin gene expression were abolished. Ceruloplasmin is not the only potential source of copper in the blood plasma. Clearly albumin and transcuprein can substitute for ceruloplasmin and maintain most aspects of normal copper distribution and homeostasis, as shown by Harris and colleagues in the ceruloplasmin knockout mouse (Meyer et al. 2001), and supported by evidence from humans lacking the active protein (Linder 2010). However, since perinatal copper transport to the fetus and newborn might be particularly sensitive to the need for this plasma protein (Lee et al. 1993), we carried out studies to examine this in the offspring of ceruloplasmin knockout mice and their wild-type siblings. Our results show that the pups of knockout mice are born with half as much copper in their livers; and the dams have less copper in their milk; but that lactation in mice (as in rats) diverts copper bound to albumin and trancuprein from liver and other organs to the mammary gland, though the magnitude of the lactational change was not as great as in the Sprague–Dawley rat. Preliminary data also suggest that reduction of copper transfer to the fetus might influence the iron content of the brain.

Materials and methods

Animals, plasma and tissue samples

Breeding pairs of heterozygous (±) ceruloplasmin knockout mice were obtained from Dr. Z. Leah Harris, Johns Hopkins University, Baltimore, MD. Matings to obtain aceruloplasminemic −/− mice and their sibling wild type +/+ controls, and use of these mice to determine how lack of ceruloplasmin affects copper metabolism have been described in detail in publications from Dr. Harris's laboratory [Harris et al. 1999]. Heterozygous mice were bred with each other, to obtain an average of 25% each of −/− and +/+ plus 50% +/− offspring. The genetic identities of the offspring were determined at 3–4 weeks of age by amplification of tail DNA by PCR, as previously described (Harris et al. 1999). Mice were ear-tagged to keep track of age and genetic ceruloplasmin status. For studies of copper transport in lactation, female −/− and +/+ mice were mated with males of the same or +/− genotype, and tissue copper and iron analyses as well as transport studies with tracer ^{64}Cu were performed on the lactating dams and their pups 2–4 days post partum. Litter sizes ranged from 8 to 14. Livers and brains of pups from a given litter (pooled in groups of 3–5) were frozen prior to wet ashing for copper and iron analyses. Milk was collected by syringe from the tips of individual teats following gentle massage applied to glands beneath, while dams were under ketamine-xylazene anesthesia, and after i.p. injection of oxytocin (1.5–2.5 U), just prior to euthanization. Blood was collected from the vena cava that bled into the thoracic cavity under subsequent pentobarbital anesthesia; plasma was obtained by centrifugation and either used fresh or after storing frozen at −20°C, in aliquots. Mice were raised and maintained on Purina rodent chow. All animal work was pre-approved by the university's IACUC.

Tissue distribution of ^{64}Cu

Uptake of copper, administered i.p. as ^{64}Cu(II)-NTA (nitrilotriacetate, at a 1:5–10 Cu:NTA molar ratio), was determined by measuring radioactivity in blood plasma, milk and organs of lactating and non-lactating female mice, 1 h after isotope injection. Injections contained <1 ng Cu and 10–30 μCi ^{64}Cu, obtained from the Mallinkrodt Institute for Radiology (MIR), Washington University, St. Louis, specific activity 20–300 μCi/μg). Lactating mice were removed from their pups 3 h before isotope injection, and milked in the last 30 min before euthanasia (from 30 to 60 min after ^{64}Cu injection). ^{64}Cu distribution data are presented as % dose, based on the recovery of radioactivity in liver, kidney, mammary gland, lung, heart, spleen, brain, skeletal muscle, and blood plasma. Total ^{64}Cu in plasma was based on cpm per ml × 0.05 body weight (in the range published for humans and rodents) (Zerounian and Linder 2002); that in skeletal muscle was calculated based on radioactivity in one thigh muscle × 11.64. (From dissection of two mice we determined that one thigh muscle comprised 8.6% of the total skeletal muscle.)

Copper and iron analyses

Samples of plasma were analyzed directly by furnace atomic absorption spectroscopy for copper concentration as previously described (Gray et al. 2009). In the case of milk and tissue samples, 10% homogenates in deionized water, were first wet-ashed with trace element-grade nitric acid. Iron in tissue and milk digests was determined with bathophenanthroline, also as previously described (Zerounian and Linder 2002).

Statistics

Calculations were performed by *t* test, P values < 0.05 for differences between means being considered significant.

Results

Effects of lactation and knocking out ceruloplasmin on the distribution of administered tracer radiocopper to liver, mammary gland and other tissues

To determine whether in lactation, the mouse like the rat diverted a major portion of incoming ionic copper from the liver directly to the mammary gland, we administered trace amounts of ^{64}Cu(II) (<1 ng) i.p. to lactating and non-lactating wild type mice, collecting milk between 30 and 60 min thereafter, and blood and tissues at 60 min. [In rats, we had shown previously that maximum uptake of ^{64}Cu tracer by liver and mammary gland had already occurred by 60 min (Donley et al. 2002)]. Figure 1 shows the results for the distribution after 60 min, as percent of dose. The data for the major organs containing the entering radioactive tracer are given in Fig. 1a. Without lactation (NL), the usual very high uptake of copper by liver and kidney was apparent (dark bars). Along with total blood plasma, these organs accounted for more than 90% of the copper administered, and most of that was in the liver. Less than 1% of the ^{64}Cu (0.9 ± 0.3) entered the mammary gland. Lactation (L) resulted in a diversion of a significant portion of the ^{64}Cu tracer directly to the mammary gland (light bars). It increased the percentage of the dose entering this organ ten-fold, to 9%, now equivalent to that in kidney or total plasma. The proportion entering the liver was reduced about 15%. The effects of lactation in the mouse were thus similar to those in the rat (Donley et al. 2002), though not as great. Proportions of radioisotope entering other organs are shown in Fig. 1b. In line with liver, enhanced uptake by mammary gland was accompanied by less isotope entering most other organs.

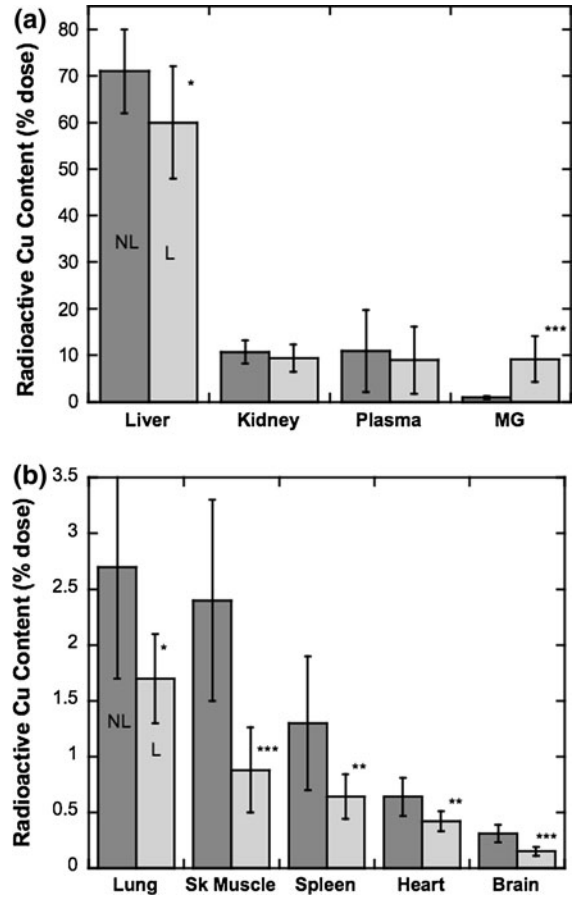

Fig. 1 Effect of lactation on distribution of ^{64}Cu to wild type (Cp+/+) mouse tissues, 1 h after injection. Nonlactating (*NL*) and lactating (*L*) Cp+/+ adult, female mice, from our colony, were injected i.p. with tracer ^{64}Cu(II)-NTA, and organs and blood were taken 1 h later. The proportions of radioactivity recovered in each organ is presented as percent of dose (Mean ± SD), based on recovery in all organs and blood (see Methods), for 7 non-lactating (*dark bars*) and 11 lactating mice (*light bars*). **a** Organs with the largest proportions of the radioactivity. *MG* mammary gland. **b** Organs with lower levels of radioactivity. Significant differences between lactating and non-lactating mice are as indicated: $*P < 0.05$; $**P \leq 0.025$; $***P < 0.001$

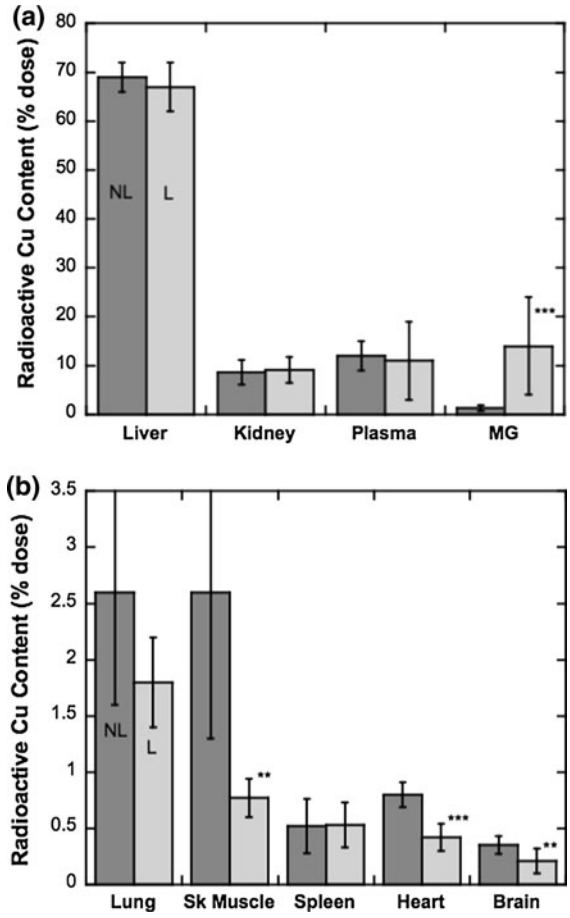

Fig. 2 Effect of lactation on distribution of ^{64}Cu to ceruloplasmin knockout mouse tissues, 1 h after injection. Data were obtained as described in Fig. 1 and are presented in the same way. Data are percent of dose (Mean ± SD) for six non-lactating (*dark bars*) and eight lactating mice (*light bars*). **a** Organs with the largest proportions of the radioactivity. *MG* mammary gland. **b** Organs with lower levels of radioactivity. Significant differences between lactating and non-lactating mice are as indicated: $*P < 0.05$; $**P \leq 0.025$; $***P < 0.001$

These responses were then examined in mice in which ceruloplasmin had been knocked out. As shown in Fig. 2, the results were very similar to those of the sibling wild type mice. Again, there was very little uptake of ^{64}Cu tracer by the mammary glands of the virgin mice (Fig. 2a; dark bars); and lactation induced a large increase in uptake (light bars), which was now of the proportion found in kidney. Other than mammary gland, most organs of the lactating knockout mice received less tracer (Fig. 2b). However, amounts in the liver were not reduced (Fig. 2a), most likely because none was being released on ceruloplasmin.

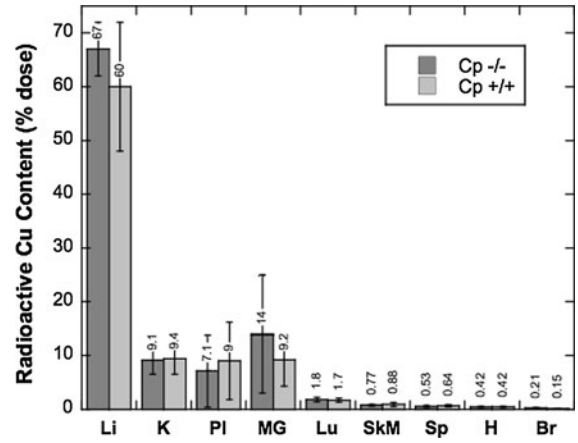

Fig. 3 Comparison of tracer copper distribution in lactating Cp (−/−) and Cp (+/+) mice. Data from Figs. 1 and 2 for lactating mice for all organs and blood plasma (Means ± SD) for 11 wild type (*dark bars*) and 8 Cp knockout (*light bars*) mice. *Li* liver, *K* kidney, *Pl* plasma, *MG* mammary gland, *Lu* lung, *SkM* skeletal muscle, *Sp* spleen, *H* heart, *Br* brain. Actual mean values are indicated above each bar. Differences between mean values for Cp (−/−) and Cp (+/+) mice were not statistically significant

A direct comparison of the tissue distributions of tracer ^{64}Cu in the lactating knockout and wild type mice is shown in Fig. 3. No statistically significant differences in tracer distribution were apparent for the Cp+/+ and Cp−/− mice. The same was the case for the non-lactating (virgin) mice (compare data in Figs. 1 and 2). Thus overall, knocking out ceruloplasmin made little difference to the initial distribution of copper that had entered the blood in ionic form, in lactation and without.

The radioactivity appearing in the milk was also measured. Two things are noteworthy. First, there was substantial radioactivity in the milk of ceruloplasmin knockout mice as well as in the wild type, indicating that the copper rapidly enters the milk of these animals as it does that of the rat (Donley et al. 2002). Second, although the results were quite variable, there was some evidence that less radioactive tracer copper was appearing in the milk when ceruloplasmin was knocked out. Figure 4a shows the ratios of radioactivity in milk (cpm/ml) relative to the concentration of ^{64}Cu in the mammary gland of the same animals (Milk/MG) and in relation to the concentration of tracer in the blood plasma (Milk/Plasma) at the 1 h time point. For the knockout mice, the ratio of radioactivity in milk relative to plasma was

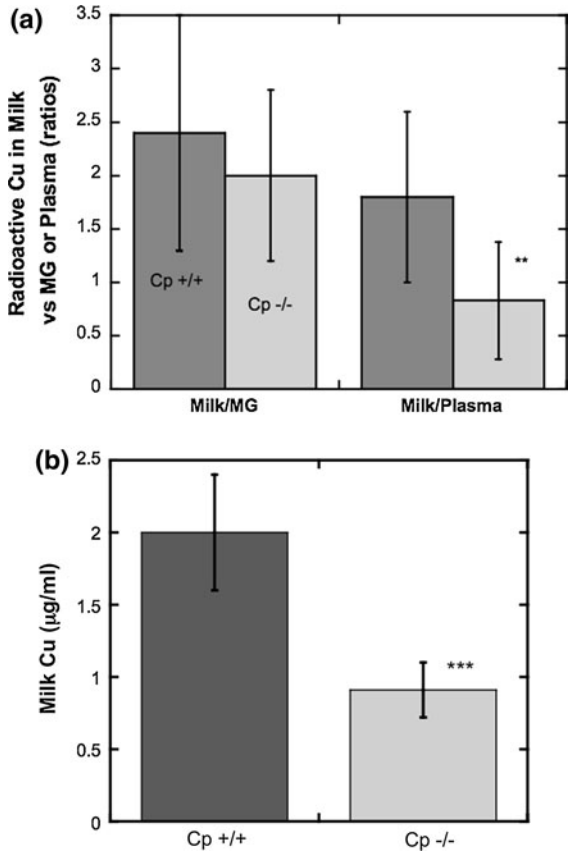

Fig. 4 Copper and ^{64}Cu tracer in milk of Cp (+/+) and Cp (−/−) mice. **a** Ratios of ^{64}Cu tracer in milk relative to that in mammary gland or plasma of the same animal 1 h after injection: ^{64}Cu cpm/ml of milk over cpm/g of mammary gland (Milk/MG), or over cpm/ml blood plasma (Milk/Plasma), for individual lactating wild type (*dark bars*) and Cp knockout mice (*light bars*). Values are Means ± SD for 7 wild type and 6 knockout mice. **b** Copper concentrations of milk (μg/ml) in 6 knockout (light bar) and 5 wild type (*dark bar*) mice; Means ± SD. $^{**}P = 0.025$ or $^{***}P < 0.001$ for the knockout versus wild type mice, as indicated

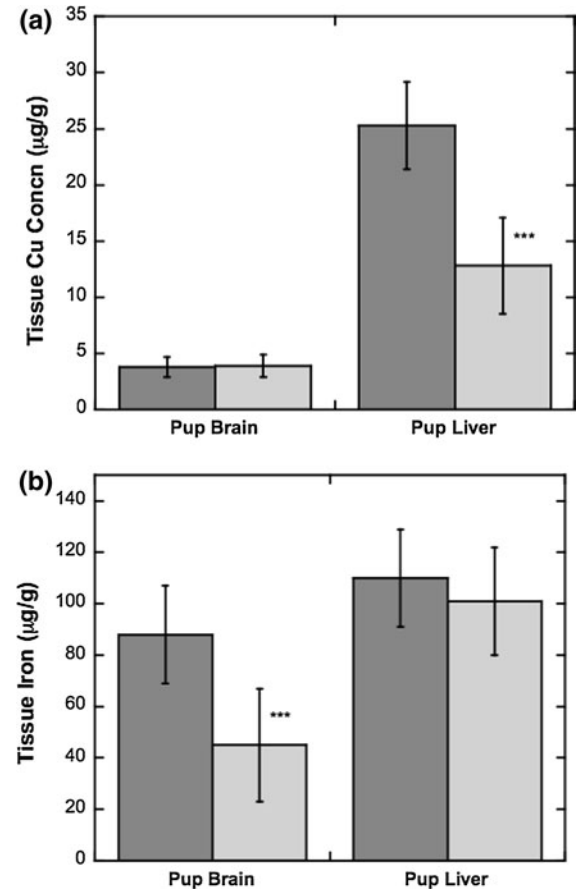

Fig. 5 Copper and iron concentrations in newborn pups of Cp knockout and wild type dams. **a** Copper concentrations (μg/g) of the brains and livers of mice 2–4 days after birth, derived from Cp (+/+) (dark bars) and Cp (−/−) dams (*light bars*). Values are Means ± SD ($N = 14$–19). **b** Iron concentrations (μg/g) of the brains and livers of mice 2–4 days after birth, derived from Cp (+/+) (*dark bars*) and Cp (−/−) dams (*light bars*). Values are Means ± SD ($N = 14$–15). $^{***}P < 0.001$ for the knockout versus wild type mice

significantly reduced; the mean ratio relative to mammary gland was a bit lower but did not reach significance.

Differences in the copper contents of milk

Milk samples from the Cp−/− and +/+ dams were also analyzed for their copper content by furnace atomic absorption spectrometry, after 10-fold dilution of whole milk in deionized water and wet washing. As shown in Fig. 4b, knocking out the ceruloplasmin gene had a marked effect, reducing copper concentrations to less than half.

Effect of knocking out ceruloplasmin on copper and iron contents of livers and brains of newborn pups

Since we had previously found in rats that plasma ceruloplasmin is the main normal source of copper for the placenta and fetus (Lee et al. 1993), we examined whether lack of ceruloplasmin expression during gestation would influence copper accumulation in vital organs of the pups. Copper contents of the livers and brains of newborn pups (2–4 days after birth) from Cp (−/−) and (+/+) dams were analyzed. As shown in Fig. 5a, the copper concentrations of pup brains

were identical. In contrast, livers of the pups from Cp knockout dams had half as much copper as those from sibling wild type dams.

Since there are interactions between copper, ceruloplasmin and iron in mammalian metabolism, and often, metal concentrations go in opposite directions (Linder 1991; Linder 2010), we also analyzed the livers and brains of the pups for iron. As shown in Fig. 5b for the liver, there was no significant difference in the iron concentration, despite a 2-fold difference in copper content (Fig. 5a). However, there appeared to be about half as much iron in the brains of the pups from the knockout mice.

Discussion

We have shown for the first time that like rats, wild type mice normally first deliver to liver and kidney most of the copper that has entered the blood in ionic form; and that lactation results in a very large increase in copper uptake by the mammary gland. In non-lactating mice, 1 h after giving tracer ^{64}Cu(II), about 80% was in liver and kidney and another 10% still in the blood. During lactation (2–5 days post-partum), the percentage in liver and kidney fell to 70%, and the proportions in mammary gland went from <1 to 9% (now equaling kidney). In the wild type mice, the drop in liver ^{64}Cu accounted for most of the increased tracer in the lactating mammary gland; but, apart from kidney and spleen, all of the other internal organs examined also took in significantly less of the isotope. Thus, in this species, not just the liver but many of the other internal organs reduced their copper uptake in favor of the mammary gland. The results for C57BL/6 background mice were similar but not identical to those obtained for Sprague–Dawley rats (Donley et al. 2002). In the latter, lactation induced a greater degree of increase in mammary gland uptake (from about 2 to about 50% of dose after 1 h); and liver and kidney accounted for most of the reduction (falling from 80 to 35% of dose). It is noteworthy that in the rats (with or without lactation) the proportion of radioisotope taken up by the kidney was much greater than in the mice; and that in the latter, lactation did not diminish the proportion entering the kidneys, whereas it did in the rats. This indicates additional subtle differences in copper metabolism between these species.

Knocking out ceruloplasmin had very little if any effect on the short term distribution of administered tracer ^{64}Cu(II) from blood to tissues, with and without lactation. One hour accumulations of ^{64}Cu in tissues were very similar, and the changes in uptakes induced by lactation were comparable. The only potential difference was that accumulation by the liver of the knockouts was not significantly lower in lactation, although most of the other tissues again absorbed less tracer. Why lactation normally reduces the copper taken up and/or retained by the liver is still unclear, but it might have to do with there being a stimulation of ceruloplasmin-copper secretion induced by hormonal changes associated with pregnancy and lactation. [Estrogen and progesterone both increase blood levels of ceruloplasmin (Linder 1991), although the effect of estrogen is not on the rate of liver ceruloplasmin synthesis (Middleton and Linder 1993), and potential effects of prolactin have not been studied.] Without the ability to form ceruloplasmin, there would be no stimulation of its secretion. Another possibility is that the copper content of the maternal livers of lactating knockout dams was higher than for the lactating wild type (and non-lactating knockout) mice, thus leading to more dilution of the entering radioisotope and a seemingly greater retention. These matters require further study.

These are the first reports on effects of knocking out ceruloplasmin on the lactating mammary gland and fetal copper accumulation in gestation. Lack of a difference in uptake of copper by mammary gland in the knockout mice is consistent with previous studies indicating little or no change in ionic copper transport in adult knockout mice or in the copper content of their tissues (Meyer et al. 2001), although mammary gland was not examined. This finding is also consistent with our previous conclusions that plasma ceruloplasmin is not the major source of copper for the mammary gland in lactation (Donley et al. 2002; Lee et al. 1993). In our rat studies with radioactive ^{67}Cu, most of the copper had entered the mammary gland from the blood plasma within the 1st h after injection (Donley et al. 2002), during which very little of the radioisotope would have had a chance to be incorporated into ceruloplasmin by the liver for secretion into blood. Radioactivity in the mammary gland declined from 2 to 4 h after injection and thereafter, when ^{67}Cu on ceruloplasmin would dominate in the plasma. However, that does not preclude its participation as an

additional copper source for this tissue (Cabrera et al. 2008), which is suggested by other data in this report (see below).

Our studies also show for the first time that, as predicted, lack of ceruloplasmin gene expression resulted in milk with much less copper. Our previous studies (Cerveza et al. 2000; Donley et al. 2002) and those of some others (Kiyosawa et al. 1995) had indicated that up to about 25% of the copper in the milk of rats and humans was due to ceruloplasmin produced by the mammary gland (Cerveza et al. 2000; Donley et al. 2002). In mice, the decrease in milk copper was about twice the expected 25%. This could be due to species differences in the proportions of copper with milk ceruloplasmin and/or a reduction in the rate of copper delivery to the mammary gland by plasma ceruloplasmin. The data comparing efficiency of transfer of exchangeable copper in the plasma (on albumin and transcuprein) to the milk, in wild type and ceruloplasmin knockout mice, were generally consistent with the difference in milk copper content, ratios of radioactivity in milk compared to plasma (vol:vol) also falling about 50%. However, as a function of the radioactivity per g of mammary gland, the proportion in milk was not comparably reduced, suggesting that the amount of tracer copper in milk did not reflect the actual copper content. This further supports the concept that plasma ceruloplasmin also conveys copper to mammary epithelial cells for the milk.

Our data on the copper concentrations in wild type mouse milk are consistent with the sparse other data in the literature for mice and rats. Our values were 2.0 μg/ml for Cp+/+ wild type mice (in the C57BL/6 background) 2–4 days into lactation. Values reported for rat milk (1.5 and 3.3 μg/ml) are in the same range (Donley et al. 2002; Suttle 1987). Somewhat higher values of 2.9–3.7 μg Cu/ml for C57BL6 mice on copper adequate diets but on the 12th day of lactation, were reported by Prohaska (1989). Mouse data for the stomach content of pups, reported by Rauch (1983) and Michalczyk et al. (2000) in terms of μg Cu/g of dry weight, translate into 1.3–1.7 μg/ml for wild type DL mice, 4–5 days post-partum, assuming a milk dry weight of about 80%, again in the same range. All of this confirms that our values of 0.9 μg Cu/ml for the milk of ceruloplasmin knockout mice (in the C57BL/6 background) are much lower. Thus, both the direct copper measurements and the radioisotope measurements support a role for ceruloplasmin in supplying copper to the milk. Nonetheless, copper entered the milk rapidly and in significant amounts, indicating that not only ceruloplasmin but other forms of copper are present in this fluid.

While ceruloplasmin appears to contribute half or more of the copper in mouse milk available to the suckling newborn, data in this report lend further credence to ceruloplasmin playing a significant role in copper transfer to the fetus. Though in general, other copper carriers in the blood plasma can substitute for ceruloplasmin to bring copper to most tissues when needed (Meyer et al. 2001), data in this manuscript show for the first time that lack of ceruloplasmin can make a real difference to the amount of copper stored in the livers of offspring before birth. At the end of gestation, fetal stores of copper in liver are very high (Linder 1991; Linder 2010). Livers of newborn rats, for example, have been reported to contain 90 μg Cu/g dry weight (Mercer and Grimes 1986) and those of human infants 300 μg Cu/g wet weight (Linder 1991). These concentrations are not normally seen again during the lifespan of the species, where in rats and humans liver concentrations are held close to 20 μg Cu/g (dry weight), or 5 μg Cu/g wet weight. Our values for the 2–4 day old mouse pups from wild type dams were 25 μg Cu/g wet weight, in rough agreement with those of others, Rauch (1983) reporting 47.8 μg Cu/g (age unspecified), and Prohaska (1984, 1989) 47–65 μg Cu/g for 11–12 day old C57BL/6 male mice. Recalculating newborn liver values of Michalczyk et al. (2000) on the basis of 25% dry weight gives 32 μg Cu/g in DL mice at 4 days of age, quite close to our values and those in another report (Allen et al. 2006). All of these pup liver copper concentrations are much higher than adult mouse liver values, reported as 2.6 μg Cu/g in 10–12 week old Cp (+/+) mice (Meyer et al. 2001), the same strain used in our studies. The pups obtained from the ceruloplasmin knockout dams had half as much liver copper as those from their wild type sibling dams, falling from 25 to 12 μg/g, a highly significant drop ($P < 0.001$). Although this decrease might have been exacerbated by suckling for a few days on milk that also had less copper, most of the effect is likely to be the result of differences in copper delivery during gestation.

Of additional interest were our findings, reported here for the first time, that the brains of the newborn mice from Cp (–/–) dams were not deficient in copper despite some reduction in copper supply during

gestation (evidenced by lower liver stores). (Our values were the same as those obtained by Meyer et al. (2001) for the same kinds of mice as adults.) That brain copper concentrations were not altered is consistent with the concept that the developing brain has preferential access to available copper and that the storage of excess copper by the liver is reduced when overall copper supplies are limited. However, we found that the iron concentrations of pup brains were reduced, suggesting that having less copper delivered from the mother during gestation might result in less being available to the brain, even though liver iron concentrations were not altered. This is interesting but must be studied further.

The studies reported here lead to the conclusion that, while plasma ceruloplasmin may not be the primary source of copper for the mammary gland, ceruloplasmin produced by the mammary gland contributes a large proportion of the copper received by the pups through the milk, and this lack of copper is not circumvented by other transport systems in the mammary epithelial cells that supply copper to milk. Ceruloplasmin plays an equally significant role in delivering copper to the fetus for storage in the liver, making more of this trace element available to the infant after birth in support of growth and formation of new tissue, for which copper is essential.

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